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Metabolite profiling of four different tissue locations in grape leaf of brown spot disease caused by *Pseudocercospora vitis*

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Abstract

Infected 'Campbell Early' grape leaf tissues were collected according to four different tissue locations of brown spot disease: center of the lesion (A), lesion border (C) the uninfected leaf tissue (D) and tissue from A and C as well as the lesion halo (B). A total of 118 metabolites were identified using polar phase GC-MS analysis with sample derivatization and annotated four different sampling tissues. Metabolites were clustered into three groups according to tissues (tissue A, tissue B, and tissues C and D) in the PCA score plot. Tissues C and D were separated in the PCA plot; therefore, they had similar metabolite contents variations. 20 of the metabolites were significantly related to tissues A and C in the S-plot of OPLS-DA. Most of the metabolites, including caffeic acid, succinic acid, and citric acid, were increased in related contents in uninfected tissues (C and D) compared with lesions (A and B). In contrast, D-mannitol, xylitol, and raffinose were increased in the lesion tissues (A and B). Neohesperidin, lactulose, and dehydroascorbic acid were found in the tissue B. As a result, those three metabolites can be expected to relate to the defense mechanism of grapes against brown leaf spot disease.

Keywords: Disease resistance; GC-MS; Metabolic profiling; Multivariate data analysis; *Vitis labruscana*.
Abbreviations: CV-ANOVA_Cross-validated analysis of variance; FAME_Fatty Acid Methyl Ester; MSTFA_N-methyl-N-(trimethylsilyl)-trifluoroacetamide; OPLS-DA_orthogonal projections to latent structures discriminant analysis; PCA_principal component analysis; PLS-Partial Least Squares; ROS_reactive oxygen; VIP_variable importance for projection.

Introduction

The grape is an important fruit used to make wine and raisins. The grape 'Campbell Early' is a most important table grape cultivar of Korean vineyards, but is susceptible to brown leaf spot disease caused by *Pseudocercospora vitis* (Kim and Shin, 1998). Brown leaf spot disease often causes defoliation of the vine that can be lead to severely decreased grape quality and vine vigor before death (Park et al., 2004; Jung et al., 2009). The disease resistance metabolites in plants are not easily recognized because a lot of primary and secondary metabolites are related to disease resistance, and the metabolic pathways are complicated and linked to each other through various outside stresses.Many previous studies have searched pathogen-specific chemicals that are known secondary metabolites. Although those secondary metabolites are important in resistance to the pathogen locally, they cannot completely explain metabolite variations on the other side of the plant. Metabolic profiling is a new, useful technique to provide clues within the whole metabolite scale (Sumner et al., 2003). One technique for studying metabolomics is GC-MS. High-resolution separations from the GC column are analyzed, and the precise electron ionizations are searched in libraries (Warren, 2013). Although the GC-MS technique is not sufficient for analysis of high molecular weight compounds, derivatization techniques could help to analysis for some part of metabolites (Knapp, 1979). Some advantages of GC-MS analysis in metabolomics are the well-organized stable protocol from sampling to data analysis and relatively broad coverage of compound classes (Lisec et al., 2006). Silylation is the most suitable derivatization method for non-volatile metabolites such as hydroxyl and amino compounds (Pierce, 1968). Some silylation agents were tested using the GC-MS platform for plants, which has high reliability and reproducibility. One silylation agent, N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was used to reveal the whole range of metabolites in plants (Fiehn et al., 2000; Roessner et al., 2000; Lisec et al., 2006). Recently, some metabolomic studies used GC-MS to evaluate disease related metabolites as bio-markers of a particular pathogen, such as downy mildew on grapes (Batovska et al., 2009), Fusarium head blight on wheat (Warth et al., 2014), blast on rice (Johns et al., 2011), bacterial blight on rice (Sana et al., 2010), and *Alternaria brassicicola* on *Arabidopsis* (Botanga et al., 2012). These previous experiments used GC-MS and multivariate data analysis to identify a new aspect for pathogen related metabolites and well-known secondary metabolites. One multivariate data analysis is principal component analysis (PCA), which is an unsupervised method that reduces the dimensionality of the variances (Ericksson et al., 2006). Plots in the multidimensional space are used to determine the similarities and differences between treatment data. Another supervised analysis, orthogonal projections to latent structures discriminant analysis (OPLS-DA), is a recent modification of the Partial Least Squares (PLS) method. This method concentrates its predictive power into the first component to provide an improved model for transparency and interpretability (Ericksson et al., 2006). Multivariate data analysis is critical in metabolomic studies for providing a significant clue to the important findings in the numerous experiments. In this study, we aimed to find the all the pathogen-specific metabolites against *Pseudocercospora vitis* in the grape cv. 'Campbell Early' using GC-MS and

multivariate data analysis. Moreover, we also attempted to identify the correlation between the variability of metabolites and disease development using two different multivariate analyzes, PCA, and OPLS-DA.

Results and Discussion

Leaf metabolite differences with unsupervised analysis (PCA)

There were 118 of metabolites on average in each replicated sample when using retention indexing in the metabolic Fiehn library. Metabolites in the different leaf tissues were plotted using PCA (Fig. 1). The leaf tissues were plotted in three different groups based on the eigenvector scores, but replicates of tissue C and D samples were plotted together. Two principal components (PC) accounted for 76.4% of total variations, PC1 for 50.7% and PC2 for 25.7%. The PCA model quality was validated based on two variables: R_2X and Q_2X. Our results revealed an R_2X value of 0.764 and a Q_2X value of 0.534. Generally, R_2X values range between 0 and 1, where 1 indicates a model with a perfect fit. Moreover, a Q_2X value > 0.5 indicates a model with good predictability and a value between 0.9-1.0 indicates a model with excellent predictability (Eriksson, 2006). In this PCA, results were used to find metabolite differences between the tissues collected. The PCA is an unsupervised method that attempts to create a model of the data without a priori information; therefore, it can provide an overview of the whole metabolite profile and find the suspected related metabolite among the samples using a qualitative analysis. Thus, PCA does not provide statistically significant evidence for finding a biomarker. In our PCA result, tissues A, B and C separated, but tissues C and D were not separated from each other (Fig. 1 A). Tissue C, from the border region of the disease symptom, was expected to contain more highly activated metabolite contents than the other tissues. Tissue C was not different from tissue D, which looked like normal tissue but was located on the same diseased leaf. Therefore, tissue D already fully produced the defense metabolite against outside invaders like tissue C. Tissue C and D samples were located in the same direction on the PCA score plot (Fig. 1 A) and most of the metabolites related to tissue C and D were weighted on the right side of the PCA loading plot (Fig. 1 B). This result indicated that most of the metabolites of tissues C and D were the same, but the contents of each tissue were different. The PCA loading plot showed that some of the metabolites related to tissue A were raffinose, xylitol, and D-mannitol (Fig. 1 B).

Leaf metabolite differences with supervised analysis (OPLS-DA)

The supervised analysis method, OPLS-DA, is similar to PLS-DA, but a single component is used as a predictor for the model and the other components describe the variation orthogonal to the first predictive component (Westerhuis et al., 2010). In this experiment, we collected different tissues from the leaf and tried to find a significant metabolite using OPLS-DA. The S-plot is generally used to identify the factors that interact with each other in the OPLS-DA model, and the results are useful for identifying a metabolite marker with statistical significance in the different conditions (Wiklund et al., 2008). Before S-plot plotting, data are cut down based on variable importance for projection (VIP) values > 1 and $p < 0.05$ to select for potentially related metabolites. The selected metabolites with VIP values > 1 were associated significantly with the separation shown in both S-plot models, as the functions calculated from the weighted sum of squares of the PLS weight indicate the importance of the selected variable to the whole model (Azizan et al., 2012). In this study, a total six different OPLS-DA models were combined for four different leaf tissue samples. Among the six different OPLS-DA models, only three were significantly different from each other based on the metabolite contents (Table 1). The first model (A vs. B) resulted in one predictive and one orthogonal (1 + 2) component with a cross-validated predictive ability Q_2(cum) of 87%, a total explained variance R_2X of 63%, and a variance related to class separation R_2 p(X) of 18%. The second model (A vs. C) resulted in one predictive and three orthogonal components (1 + 2). The predictive ability Q_2(cum) was 95%, the total explained variance R_2X was 69%, and the variance related to the differences between the two classes R_2 p(X) was 14%. The third model (A vs. D) resulted in one predictive and three orthogonal components (1 + 2). The predictive ability Q_2(cum) was 90%, the total explained variance R_2X was 67%, and the variance related to the differences between the two classes R_2 p(X) was 13% (Table 1). Another three models demonstrated the none of significance into the cross-validated analysis of variance (CV-ANOVA) ($p < 0.05$) and were not clearly separated from each other in the PCA score plot (Fig. 1). In general, values of R_2 and Q_2 > 50% are considered satisfactory for metabolic experiments (Azizan, 2012), but CV-ANOVA uses a statistical diagnosis method in the PLS and OPLS models (Eriksson et al., 2008). Despite that the R_2X and Q_2(cum) were > 50%, models B vs. C and B vs. D showed no significance ($p < 0.05$) in their differences according to CV-ANOVA. We can find a meaningful metabolite to compare laboriously each metabolite with using an ANOVA test in PCA, but the CV-ANOVA in the OPLS model can help to find a metabolite that is statistically significant between models.

Disease related metabolites

A comparison between tissues A (lesion) and C (border of the lesion) was expected to find a metabolite that was closely linked to brown spot disease in the grape 'Campbell Early'. According to the A vs. C model on the OPLS-DA, a total of 20 metabolites were significantly related to the symptom lesion and border of the lesion (Fig. 2). Of those metabolites, 17 were decreased in the tissue A from the lesion. These metabolites were classified as amino acids, sugars, and organic acids and included D-glucose-6-phosphate, D-allose, fructose, L-glutamic acid, succinic acid, citric acid, D-malic acid, caffeic acid, phosphoric acid, gluconic acid, glyceric acid, catechin, galactinol, epicatechin, and purine-riboside. Most metabolites were included in the basic metabolic pathway as a part of the plant defense system. Both catechin and epicatechin have been reported as part of the grape's disease resistance. D-glucose-6-phosphate is a precursor for hypersensitive reaction and reactive oxygen species (ROS) production against the pathogen (Asai et al., 2011). In addition, D-allose acts a triggering molecule in the pathogen attack (Kano et al., 2013) and gluconic acid has antimicrobial activity and regulates other antimicrobial compound production (Werra et al., 2009).

In contrast, three chemicals, D-mannitol, xylitol, and raffinose, were significantly increased in the center of the lesion. Those chemicals were found similarly to the PCA result (Fig. 1 B). Those chemicals were previously reported to be related to plant and microbe interactions; for example, raffinose is an oligosaccharide that accumulates in plant cells in response to

Table 1. Cross-validation of OPLS-DA models at the tissue compares combination models collected different leaf tissues from grape 'Campbell Early' leaves infected by *Pseudocercospora vitis*.

Model[a]	Fitting values				CV-ANOVA
	Component[b]	R_2X	R_2Y	$Q_{2(cum)}$	*p*
A vs. B	(1+2)	0.630	0.987	0.877	0.048
A vs. C	(1+2)	0.691	0.995	0.949	0.010
A vs. D	(1+2)	0.669	0.990	0.895	0.043
B vs. C	(1+3)	0.548	0.994	0.691	0.296
B vs. D	(1+5)	0.560	0.993	0.524	0.560
C vs. D	(1+3)	0.612	0.992	0.228	0.976

[a] Center of the lesion (A), lesion, a halo of lesion border, and lesion border of the leaf (B), lesion border (C) and not infected leaf tissue (D). Values were calculated with Simca-P [b] Component number (predictive + orthogonal).

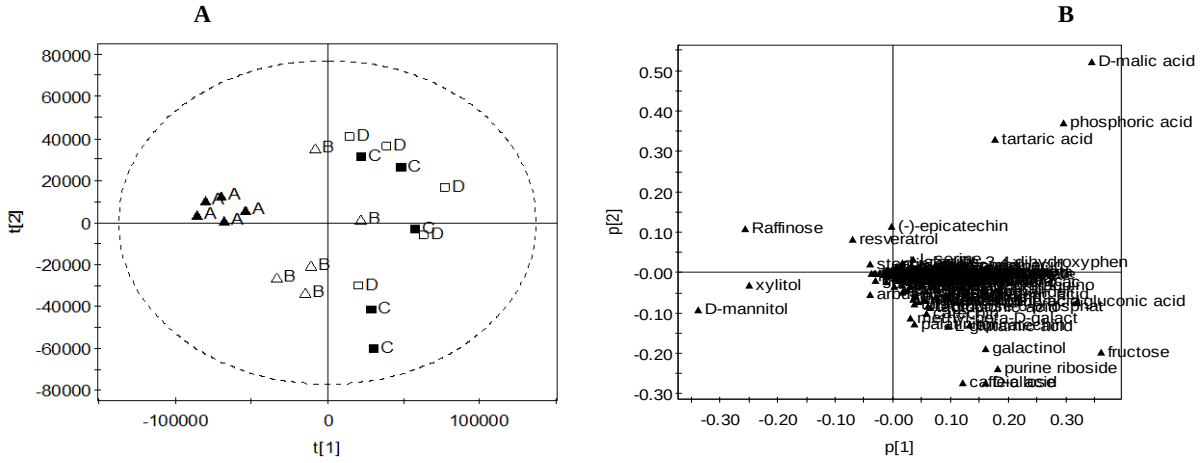

Fig 1. Principal component analysis (PCA) plots of four different locations of the leaf tissue of 'Campbell Early' grape infected by *Pseudocecospora vitis*. A. Score plot PC1 (50.7%), PC2 (25.7%), B. loading plot showed just three metabolites weighted to tissue A, but lots of chemicals weighted tissue C and D.

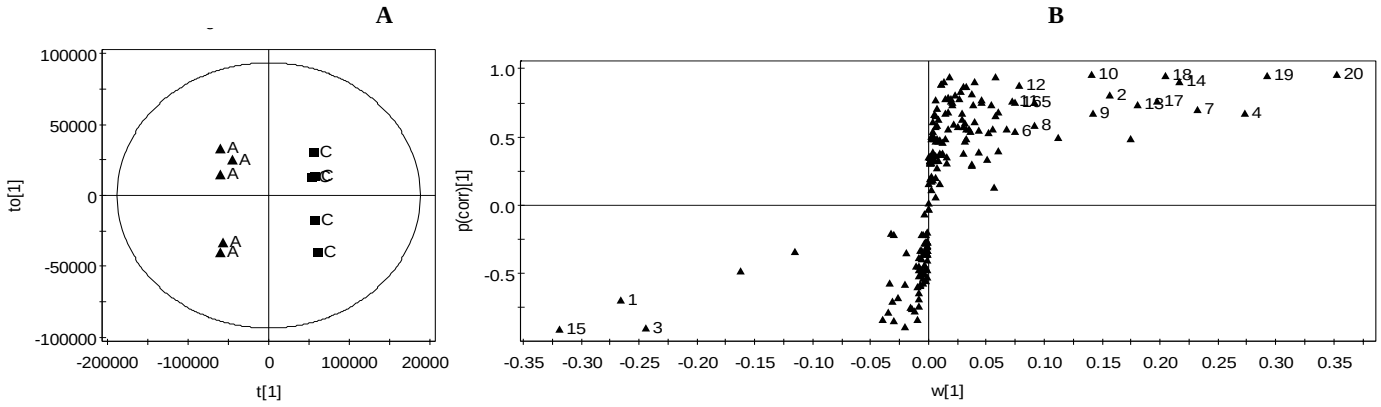

Fig 2. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) plots of compare with two of grape 'Campbell Early' leaf tissues infected by *Pseudocecospora vitis*. A. Score plot of OPLS-DA, Variation explained between tissues (69%), within tissue (14%), B. S-plot of OPLS-DA, The numbered metabolites are satisfied condition (VIP > 1 and *p* (corr) ≥ |0.5|), 1. Raffinose, 2. Galactinol, 3. Xylitol, 4. D-malic acid, 5. L-glutamic acid, 6. Lactose, 7. Phosphoric acid, 8. Palatinitol, 9. Caffeic acid, 10. Glyceric acid, 11. Succinic acid, 12. D-glucose-6-phosphate, 13. Purine riboside, 14. D-allose, 15. D-mannitol, 16. Catechin, 17. Epicatechin, 18. Citric acid, 19. Gluconic acid, 20. Fructose. On the left side numbered chemicals were significantly correlated to tissue A, and numbered chemicals on the right side were correlated to tissue C.

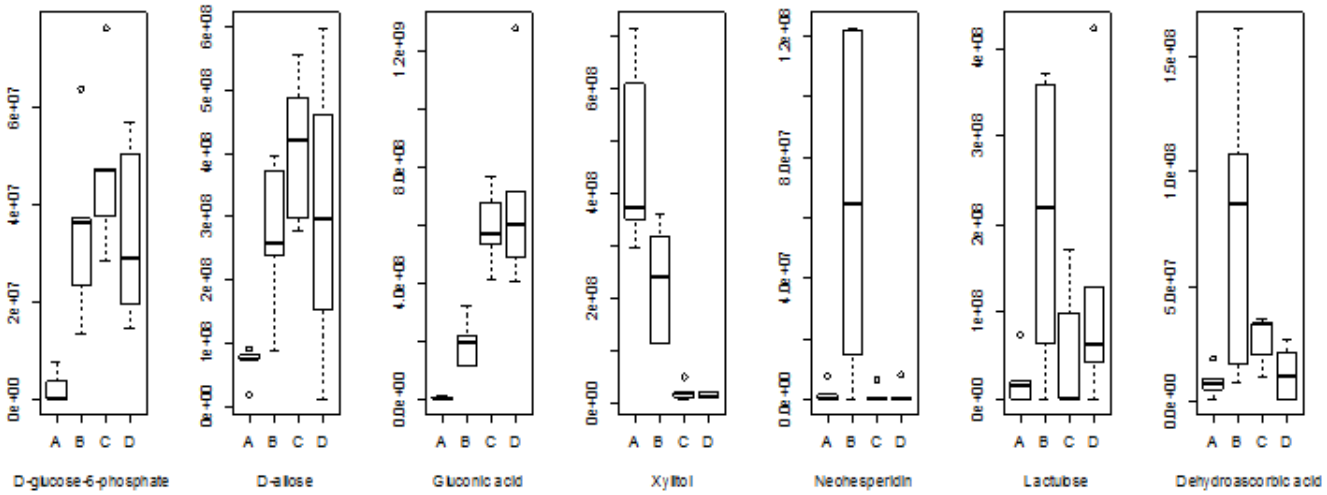

Fig 3. Putative metabolites significantly related to the disease were compared with different leaf tissue locations in the grape 'Campbell Early' infected by *Pseudocecospora vitis*. Y axis is a related area in GC-MS peak; X-axis is different infected leaf tissues.

environmental stresses (Zhou et al., 2012) and D-mannitol is used to suppress ROS-mediated plant defenses by phytopathogenic fungi (Jennings et al., 1998). The role of xylitol in the plant defense mechanism in grapes is unclear at this time, but it is well-known as an inhibitor of various bacteria.

On the other hand, we used S-plot analysis to find a precise metabolite to tissue B, by comparisons between models (A vs. B and A vs. C). Tissue B contained a yellow halo, which was expected when a specific metabolite had an intense resistance response against the pathogen. We excluded a common metabolite between models A vs. B and models A vs. C and then collected three tissue B-specific metabolites: neohesperidin, lactulose, and dehydroascorbic acid (Fig. 3). Those metabolites were highly present in tissue B, including in the halo of the lesion, and are expected to have a strong defense against the pathogen. In the oxidation caused by ultraviolet light in plants, L-ascorbate acts as an anti-oxidant by absorbing active oxygen and turning over dehydroascorbic acid (Parsons and Fry, 2012). In a previous study, the dehydroascorbic acid level was higher in *Arabidopsis* infected with pathogen fungi *Alternaria brassicicola* compared with *Arabidopsis* that was mock inoculated after 24 hours (Botanga et al., 2012). Neohesperidin is an isoflavone, which accumulates to considerable levels in the leaves and fruit of citrus species; although, the biological role in plants is not clear (Frydman et al., 2005). However, lactulose is converted from lactose by beta-galactosidase (Lee et al. 2004), but, unfortunately, there is little known about the role of lactulose in plant disease resistance. These metabolites are expected to be specific for the grape 'Campbell Early' and brown spot disease caused by *Pseudocercospora vitis*. *Cercospora* is closely related to the genus *Pseudocercospora*, and the pathogenicity and disease mechanism in their host have been studied. *Cercospora* spp. produces a photosensitizing fungal toxin cercosporin, which makes singlet oxygen ($^1O_2$) in light (Daub and Hangarter, 1983). As a result, the activated oxygen caused peroxidation of the host plant cell membrane lipids. Finally, nutrients are leaked from the broken host cell membrane (Daub and Ehrenshaft, 2000). In our result showed that both dehydroascorbic acid and neohesperidin have predicted roles in plant disease resistance such as antioxidant process. In conclusion, some metabolites have already been reported to have a part in the defense mechanism in plants, and some have been identified through the statistical analysis. Our results suggested tissue specific metabolites that may have the potential for use as biomarkers, and may provide meaningful information for a different line of research.

Materials and Methods

Plant materials

Grape 'Campbell Early' leaves which were clearly developed lesion was collected in the experimental vineyard Suwon Korea at harvest season in 2014. Cleaning and removing contamination of leaf surface with an alcohol soaked paper towel. Leaf tissues were separated to four different tissues on the leaf disease lesion: center of the lesion (A), lesion border (C) the uninfected leaf tissue (D) and tissue from A and C as well as the lesion halo (B). Each leaf tissue sample was made 5 mm leaf disk with a punch immediately put on the liquid nitrogen. Each part of 20 leaf disk tissue was grinded on a mortar with LN_2. Each tissue sample was prepared five replicate, and each 50 mg of powder was transfer into a 1.5 ml tube. Grinded samples were kept at -80℃ deep freezer and analyzed within one week.

Sample extraction

Sample preparation method was use modified to Weckwerth (2004). Metabolite extraction was obtained from sampling tube in 1 ml of extraction buffer degassed methanol, chloroform, water, (5:2:2, v/v/v) and shaking 5 minutes at 4℃. Extracts centrifuged at 20,000 rpm, the supernatant was transferred new tube, and dried in speed vacuum dryer for 5 hours (Bioneer, Korea). For derivatization, added 20 µl of methoxyamine solution, methoxyamine (Sigma 226904, USA) 20 mg^{-1}·ml pyridine (Sigma 270407, USA) in dried sample tubes and shaking for 90 minutes at 28℃. 180 µl MSTFA (Fluka 68768, Swiss) and 10 µl FAME marker (Supelco C8-C24, USA), use standard retention time marker, were added in each tubes and then shaking for 30 minutes at 37℃. Each prepared sample was transfer auto-sampler vial with insert (Supelco #24722, USA).

GC-MS analyze

GC-MS system consisted of a gas chromatograph (Agilent

6890, USA) and mass spectrometer (Agilent 5985, USA). 1 μl of sample was injected into a splitless mode, and operating at a temperature of 230 °C at a helium carrier gas flow rate of 1 ml min^{-1}. The column used an HP-5MS, 5% phenyl methyl siloxane. The temperature was 3 min heating at 80°C followed by a 5°C min^{-1} and final 8 min heating at 280°C. Separated ion was detected MS detector at 250°C and recorded at two scans per sec with an m/z 50-600 scan range. One blank sample and One QC samples were injected at the start of an analytical sequence. The analysis order was randomly composed of samples. Data files after GC-MS analysis were treated with deconvolution process by AMDIS (Agilent, USA) with standard parameter (component width = 12; model Ion 0, 73, 207, 281; resolution, sensitivity and shape requirements = medium). Retention time variation adjusted compare to FAME marker as a retention time standard in a process inside AMDIS program. Peak annotation results were exported text files matched metabolite in the DB. Peak annotation results were matched metabolite in the metabolomics Fiehn DB (Agilent G1676AA, USA) and exported to text files (Kind et al., 2009)

Multivariate analysis

For statistical analysis, each data was treated rescaling which divided by the square root of standard deviation (Pareto scaling; Van den Berg et al., 2006) of each sample variance. Statistical analysis of metabolic profiling with principal component analysis (PCA), and orthogonal projections to latent structures (OPLS) were analyzed by the SIMCA-P+ software (v12.0, Umetrics, Umea, Sweden; Ericksson et al., 2006).

Conclusion

Disease related chemicals in the plants are reported to be related to the phenolic compounds. In our experiment, some non-phenol compounds revealed different contents in the location of a disease symptom lesion. This result suggests that plant defensive mechanisms against a pathogen are related to total metabolic pathways. Whole metabolite profiling and multivariate statistical analysis are useful tools for an overview of total metabolic pathway processes in various biotic and abiotic stresses. Therefore, that can provide us critical clues for increasingly detailed studies.

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